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Diet-induced obesity alters protein synthesis: Tissue-specific effects in fasted vs. fed mice

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Abstract

The influence of obesity on protein dynamics is not clearly understood. We have designed experiments to test the hypothesis that obesity impairs the stimulation of tissue-specific protein synthesis following nutrient ingestion. C57BL/6J mice were randomized into two groups: group 1 (control, n = 16) were fed a low-fat, high-carbohydrate diet and group 2 (experimental, n = 16) were fed a high-fat, low-carbohydrate diet *ad libitum* for 9 weeks. On the experiment day, all mice were fasted for 6 hours and given an intraperitoneal injection of ²H₂O, they were then randomized into two sub-groups and either given a sham saline gavage or a liquid-meal challenge. Rates of protein synthesis were determined *via* the incorporation of [²H]alanine (5 hours post-challenge) into total gastrocnemius muscle protein, total liver protein and plasma albumin. High fat feeding led to an increase in total body fat (p < 0.001) and epididymal fat pad weights (p < 0.001) and elevated fasting plasma glucose levels (p < 0.01). Diet-induced obesity (i) did not affect basal rates of skeletal muscle protein synthesis, but did impair the activation of skeletal muscle protein synthesis in response to nutrient ingestion (p < 0.05) and (ii) slightly reduced basal rates of synthesis of total hepatic proteins and plasma albumin (p = 0.10), but did not affect the synthesis of either in response to the meal challenge. In conclusion, there are alterations in tissue-specific protein metabolism in the C57BL/6J mouse model of diet-induced obesity. This model may prove to be helpful in future studies that explore the mechanisms that account for altered protein dynamics in obesity.

Keywords

protein metabolism; mouse models; muscle; liver; type 2 diabetes

Introduction

The incidence of obesity and its accompanying morbidities has reached epidemic proportions, creating major healthcare challenges and costs^{1,2}. The association of a variety of endocrine alterations and changes in the concentration of circulating hormones typically seen with obesity has led to the description of a metabolic syndrome, characterized by hyperinsulinemia, glucose intolerance, dyslipidemia, hypertension, and increased risk of diabetes and coronary heart disease. Although there is general agreement regarding the association between obesity and impaired regulation of carbohydrate and lipid metabolism the influence of obesity on protein

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metabolism is somewhat controversial³⁻⁶. Some studies have found significant differences in protein metabolism in obese vs. non-obese human subjects⁶⁻⁸, whereas other studies have not^{5;9;10}.

One possible explanation for some of the apparent differences in protein metabolism may be found in the study of Jensen *et al*⁷. Namely, they examined whether obesity was associated with abnormalities in leucine turnover in the post-absorptive state in age-matched pre-menopausal women. The obese women had increased whole-body proteolysis, as measured by leucine carbon flux, compared with non-obese women. In addition, differences in body fat distribution, i.e. upper body vs. lower body obesity, were associated with abnormalities in protein metabolism (upper body obesity impaired the anti-proteolytic response to insulin when compared to lower body obesity and non-obese women)⁷. Thus, the location of the excess body fat plays an important role.

The observations reported above are consistent with the hypothesis that insulin's action, as an anabolic hormone on suppressing protein breakdown and stimulating protein synthesis, could be impaired in obesity. However, conflicting data have been obtained from studies that have used the insulin-clamp method in combination with isotope tracers. Insight into a possible explanation of the apparent discrepancies may be found in the work of DeFronzo and colleagues. For example, Luzi *et al*⁸ demonstrated that although proteolysis is sensitive to regulation *via* insulin, the dose of insulin affects the conclusions that are drawn, e.g. certain differences between obese vs. control subjects were observed at a low dose of insulin but not at a high dose of insulin. Those studies suggest that the dose-response (i.e. insulin-proteolysis) requires attention, and that one may overcome certain defects depending on the experimental design. In addition, although glucose production and lipolysis can be suppressed $\sim 100\%$ when high doses of insulin are infused (e.g. $\sim 40 \text{ mU insulin} \times \text{m}^{-2} \times \text{min}^{-1}$)¹¹, it appears that maximal suppression of proteolysis (which also occurs at an infusion rate of $\sim 40 \text{ mU insulin} \times \text{m}^{-2} \times \text{min}^{-1}$) only results in a $\sim 25\%$ reduction in endogenous leucine flux¹². Consequently, it may be that certain discrepancies in the literature arise from (i) the narrow apparent range of insulin sensitivity of proteolysis, (ii) the fact that high doses of insulin may mask subtle defects in insulin action and (iii) the possible heterogeneity within the obese population.

As with studies of proteolysis, the interpretation of studies regarding insulin-mediated stimulation of protein synthesis warrants caution since protein synthesis requires the presence of amino acid substrates. For example, Tessari *et al*¹³ found that both hyperinsulinemia and hyperaminoacidemia were required to stimulate net leucine deposition into body protein in postabsorptive healthy subjects. Namely, hyperinsulinemia decreased endogenous leucine Ra (i.e. proteolysis), whereas hyperaminoacidemia (alone or in combination with hyperinsulinemia) increased leucine Ra¹³. Chevalier *et al*¹⁴ recognized this point and used an "insulin and amino acid clamp" to study protein turnover in obese vs. lean women. They demonstrated that protein catabolism was equally suppressed in both obese and lean women, however, protein synthesis was less stimulated in the obese group, as well, the amino acid infusion rates required to maintain baseline levels were also lower¹⁴.

In reviewing the literature on protein turnover in obesity we found that a substantial number of investigators have relied on measurements of leucine flux¹⁵ in either a basal state or during an insulin-clamp $\pm$ amino acids. Presumably the controversies regarding protein dynamics in obesity are not related to limitations in the method(s), since most studies that we have reviewed used the same tracer, e.g. carbon-labeled leucine. Although measurement of leucine flux during a clamp provides unique insight since one can independently study physiological parameters, e.g. test the effect(s) of insulin vs. amino acids, an important and unaddressed question centers on whether there is (ab)normal protein synthesis following a meal in obese vs. lean subjects? Therefore, we initiated a study to contrast protein synthesis in the fasted vs. the fed state and

to determine whether the response(s) to a mixed-meal is impaired. Attention was directed towards measuring protein synthesis in skeletal muscle and liver (including plasma albumin) since the synthesis of these proteins is generally most responsive to nutritional status¹⁶. The use of labeled leucine is difficult under these conditions since the bolus of food will perturb the steady-state isotope labeling, therefore, rates of protein synthesis were determined using $^2\text{H}_2\text{O}$, a newly developed method by our laboratory that is well-suited for studying the response to an acute perturbation, e.g. feeding¹⁷.

Materials and Methods

Supplies

Unless noted, chemicals and reagents were purchased from Sigma-Aldrich. $^2\text{H}_2\text{O}$ was purchased from Cambridge Isotopes (Andover, MA). Gas chromatography and mass spectrometry supplies were purchased from Agilent Technologies (Wilmington, DE). Diets #D12450B (70% carbohydrate, 20% protein and 10% fat) and #D12451 (35% carbohydrate, 20% protein and 45% fat) were purchased from Research Diets (New Brunswick, NJ).

Biological

Male C57BL/6J mice (~ 14 grams) were purchased from Jackson Labs (Bar Harbor, ME) and randomized into 2 groups (n = 16 per group). Group 1 (controls) was fed a low-fat, high-carbohydrate (LF) diet whereas Group 2 (experimentals) was fed a high-fat, low-carbohydrate (HF) diet *ad libitum* for 9 weeks. Mice were housed 4 per cage. On the experimental day, food was removed from all cages (t = 0 min), at t = 180 min all mice were given an intraperitoneal injection of ^2H -labeled saline (0.50 ml). At 90 min post $^2\text{H}_2\text{O}$ (t = 270 min), 8 mice from each diet group were given a saline gavage (0.75 ml, sham), the remaining 8 mice from each group were given a substrate gavage (0.75 ml of a liquid meal calculated to deliver 3.75 kcal and consisting of 19% fat, 53% carbohydrate, and 25% protein; prepared by mixing soybean oil, Nestle Carnation® evaporated milk, Nestle Carnation® sweetened condensed milk, potato starch, Beneprotein® and egg albumin). At 570 min (i.e. 5 hr post-gavage) mice were sedated using isoflurane, blood was collected *via* cardiac puncture and epididymal fat pads, liver and skeletal muscle (gastrocnemius) were dissected and quick-frozen in liquid nitrogen, and plasma was isolated and frozen. The rationale behind quantifying protein synthesis over 5 hours was based on a previous study in which we found that albumin synthesis is stimulated for several hours after a meal¹⁷. Rates of skeletal muscle protein synthesis were also measured in that experiment and found to yield a similar time-dependent response as plasma albumin (not shown). This study was approved by, and conducted in compliance with the policies of, the CWRU Institutional Animal Care and Use Committee.

Analytical

Food intake and body weights were measured weekly. Caloric intake was calculated by multiplying the g of food consumed by the caloric density of each diet, i.e. 3.8 kcal per g low-fat diet vs. 4.7 kcal per g high-fat diet.

Body composition was determined *via* dissection of epididymal fat pads and ^2H -dilution. Briefly, the ^2H -labeling of body water was determined as described by McCabe *et al.*¹⁸; the ^2H -dilution yields a direct measure of water mass, from which one can estimate total lean mass and fat mass.

The concentration of plasma glucose was determined in the fasted mice (i.e. those given the sham saline gavage) using stable isotope dilution. A known volume of plasma (5 μl) was spiked with a known quantity of [6,6- $^2\text{H}_2$]glucose (5 μl of a 1mg per ml solution), samples were then deproteinized *via* the addition of 10 volumes of methanol (100 μl). The supernatant was

evaporated to dryness, reacted to form the “oxime-TMS” derivative and analyzed by GC-MS under electron impact ionization, the concentration of glucose was determined from the ratio of m/z 319 to 321¹⁹.

The fractional rates of protein synthesis in liver, plasma albumin and skeletal muscle were determined from the incorporation of [²H]alanine using a precursor:product relationship. Briefly, samples were homogenized in trichloroacetic acid (TCA, 0.1 g of tissue in 1000 μ l of 10% TCA, w/v) and centrifuged for 10 min at 4000 rpm. The protein pellet was washed twice with 5% TCA and then hydrolyzed for 20 h in 1 ml of 6N HCl at 100°C. To determine rates of plasma albumin synthesis, ~ 200 μ l of plasma was treated with 1 ml of 10% TCA. The protein pellet was washed twice with 5% TCA, albumin was then extracted from the pellet into 100% ethanol¹⁷. Following the evaporation of ethanol, samples were hydrolyzed in 1 ml of 6N HCl at 100°C.

An aliquot of a hydrolyzed protein sample was dried by vacuum centrifuge for 30 to 60 min. The samples were then reacted to form the “methyl-8” derivative of alanine, made by mixing acetonitrile, methanol and “Methyl-8” reagent (Pierce, Rockford, IL; 1:2:3, v:v:v) and heating the sample at 75°C for 30 min¹⁷. The sample was transferred to a GC-MS vial and analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system. A DB17-MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) was used in all assays. The initial temperature program was set at 90°C and hold for 5 min, increased by 5°C per min to 130°C, increased by 40°C per min to 240°C and hold for 5 min, with a helium flow of 1 ml per min. Alanine elutes at ~ 12 min. The mass spectrometer was operated in the electron impact mode. Selective ion monitoring of m/z 99 and 100 (total ²H-labeling of alanine) was performed using a dwell time of 10 ms per ion.

Calculations

Protein synthetic rate—The rate of protein synthesis was calculated using the equation:

$$^2\text{H-labeling protein-derived alanine (\%)} / [^2\text{H-labeling body water (\%)} \times 3.7 \times \text{time (h)}]$$

where the factor 3.7 represents an incomplete exchange of ²H between body water and alanine, i.e. 3.7 of the 4 carbon-bound hydrogens of alanine exchange with water^{17;20}. This equation assumes that the ²H-labeling in body water equilibrates with free alanine more rapidly than alanine is incorporated into newly made protein and that protein synthesis is linear over the study²¹.

The percent change of protein synthesis in various samples was determined by comparing the individual mice in an experimental group (e.g. liquid meal gavage, fed group) against the mean of the respective control group (e.g. sham saline gavage, fasted group) using the equation:

$$[(\text{fed}_{\text{mouse } x} - \text{fasted}_{\text{mean}}) / \text{fasted}_{\text{mean}}] \times 100$$

where “fed_{mouse x}” refers to a single mouse in the fed group. The mean $\pm$ sem was calculated.

Statistics

The sample size required for this study was estimated by performing power calculations (5% level of significance with 80% power) using data acquired in experiments with a similar design in which we measured protein synthesis in basal and stimulated states. Unless noted, data are expressed as mean $\pm$ sem. Comparisons of various parameters (e.g. body mass, percent fat, blood glucose concentration) between low-fat fed vs. high-fat fed mice were made using one-tailed t-tests assuming equal variance and the effects of nutritional state (fed vs. fasted) and

obesity (low-fat vs. high-fat diet) on protein synthesis were determined using a two-way ANOVA (SPSS v. 14.0).

Results

Figure 1 demonstrates that regardless of the dietary composition, total caloric intake was similar between the mice in each group when expressed as kcal consumed per mouse per day (Figure 1, Panel A). For example, significant differences were only observed over two of the experimental weeks, also, the cumulative caloric intake was similar when data are expressed as kcal consumed per mouse over the course of the study, i.e. 503 ± 6 vs. 518 ± 16 total kcal per mouse, LF vs. HF diet, respectively ($p = 0.134$). Consumption of the HF diet, as compared to the LF diet, promoted a greater increase in body mass (Figure 1, Panel B) which was related to an increase in total body fat and epididymal fat, $\sim 65\%$ and $\sim 92\%$ increases, respectively, i.e. total body fat accounted for $28.1 \pm 1.7\%$ vs. $17.3 \pm 1.9\%$ of body weight ($n = 16$ each, $p < 0.001$) and epididymal fat reached 1.00 ± 0.08 g vs. 0.52 ± 0.02 g ($n = 16$ each, $p < 0.001$) in HF vs. LF fed mice, respectively; total lean body mass was similar in the two groups, 20.4 ± 0.8 g vs. 21.4 ± 0.7 g in HF vs. LF fed mice, respectively ($n = 16$ each, $p = 0.151$).

Mice on the HF diet developed elevated fasting plasma glucose, 8.85 ± 0.37 mM vs. 7.05 ± 0.19 mM in mice on the LF diet ($n = 5$ to 7 each, $p < 0.01$). Although we were not able to measure plasma insulin concentrations in this study, we have measured fasting plasma insulin in mice fed the respective diets for 1, 4 and 12 weeks²². In that study we found no difference after 1 week of dietary intervention (i.e. ~ 60 pM in each diet group), whereas fasting plasma insulin was doubled ($p < 0.01$) in HF vs. LF fed mice after 4 and 12 weeks of intervention, i.e. ~ 113 vs. ~ 66 pM and 140 vs. 75 pM, respectively. These observations support the development of a model of diet-induced obesity with impaired fasting glucose.

Figure 2 demonstrates the fractional rates of protein synthesis in total muscle and liver proteins and plasma albumin. The relative differences in basal values between muscle and liver and the relative agreement between liver and plasma albumin are in accord with the literature²³. Also, consistent with other reports one would expect a stimulation of protein synthesis in fed (liquid meal) vs. fasted (sham saline) mice, as we observed¹⁷. These data demonstrate that diet-induced obesity leads to an impaired activation of skeletal muscle protein synthesis (Figure 2, Panel A, an increase of $95 \pm 18\%$ in LF fed mice vs. $43 \pm 7\%$ in HF fed mice, respectively). Although there is no apparent effect on the stimulation of total liver protein synthesis or plasma albumin (Figure 2, Panel B an increase of $16 \pm 3\%$ in LF fed mice vs. $21 \pm 3\%$ in HF fed mice and Panel C an increase of $38 \pm 3\%$ in LF fed mice vs. $44 \pm 4\%$ in HF fed mice, respectively), there is a slight reduction in basal hepatic protein synthesis and albumin synthesis.

Discussion

Since obesity is typically associated with insulin resistance and since insulin regulates protein dynamics, it is not unreasonable to suspect that obesity would alter protein synthesis. However, the literature regarding the relationship between obesity and protein metabolism is somewhat controversial. The primary aim of our study was to quantify rates of protein synthesis in obesity. Although we considered various metabolic models we decided to use the high-fat fed C57BL/6J mouse vs. a genetic model (e.g. the ob/ob mouse) since one can examine the time-course for the induction of obesity and related diseases (e.g. type 2 diabetes) and/or the reversal of obesity *via* changes in the macronutrient composition of the diet^{24;25}. In addition, since this strain is often used as the background onto which genetic modulations are introduced it is possible to address questions regarding interactions between diet and genetics. We also considered the experimental conditions, e.g. should studies be performed in a basal and/or a stimulated state? We chose to contrast fasting vs. feeding (as compared to an insulin-glucose

clamp) to simulate a more reasonable physiological scenario(s). Since we used an oral gavage to deliver the liquid meal, special attention was given to minimize the stress. For example, mice were briefly sedated before the gavage was given and the controls (fasted groups) were given a sham gavage of saline.

It is of interest to note that in the seminal studies of Surwit and colleagues on diet-induced obesity in C57BL/6J mice the data are often expressed as a “feed efficiency” (i.e. g of fat gained per kcal consumed)²⁵. Over the course of our studies²⁶, we became intrigued by the fact that when C57BL/6J mice are given *ad libitum* access to either a HF or a LF diet, animals typically consume a similar amount of calories yet the phenotypes are widely different. As expected, in the current study we observed a significant change in body composition in mice that were fed the HF vs. the LF diet, there was approximately a doubling of total body fat and epididymal fat (there was no difference in total lean mass). We were surprised by the fact that the total caloric intake was only slightly different over the course of the study (Figure 1A, HF fed mice consumed ~ 15 kcal more than LF fed mice over the course of the study). Although one would not require a large caloric imbalance to explain the fact that the final difference in body mass was ~ 2.5 g in HF vs. LF fed mice, other factors could influence the development of obesity including (i) the patterns of food intake (e.g. small frequent meals vs. larger boluses of food), (ii) the partitioning of nutrients following a meal and/or (iii) altered energy expenditure (e.g. a difference in activity)²⁵. For purposes of the current study, the physiological/biochemical mechanisms that account for the development of obesity are not important, nevertheless these observations suggest the merit of future studies in C57BL/6J mice to obtain new knowledge regarding the pathogenesis of diet-induced obesity. Namely, the development of obesity in C57BL/6J mice is clearly not characterized by overt hyperphagia but more likely by some subtle factor(s)²⁵. A careful dissection of the etiology of diet-induced obesity in this model may improve our understanding regarding fundamental causes of excess weight gain.

In the current study, attention was directed towards quantifying the synthesis of total protein in skeletal muscle and liver (including plasma albumin) since these end-products are sensitive to nutritional status²⁷ and since these sites constitute major depots for protein following a meal¹⁶. For example, one can estimate that ~ 25% of the increase in whole-body protein synthesis during feeding is related to an increase in albumin synthesis²⁸ and a substantial fraction of the remainder is related to an increase in the synthesis of skeletal muscle protein¹⁶. Consistent with the literature, we observed a stimulation of protein synthesis in fed vs. fasted mice regardless of the pool that was studied (Figure 2), e.g. a 2-fold increase in muscle protein synthesis after a meal was reported by others²⁹.

Our data demonstrate that diet-induced obesity leads to an impaired stimulation of skeletal muscle protein synthesis (Figure 2, Panel A, an increase of ~ 95% in LF fed mice vs. ~ 43% in HF fed mice, respectively). Although this observation is intriguing, we cannot state whether this is a direct and/or an indirect effect, i.e. whether there is an impaired activation of translation initiation and/or whether there is an altered digestion/absorption of the meal. Also, it has been speculated that if skeletal muscle protein degradation is likely to be constant from day-to-day under normal physiologic conditions, then the activation of protein synthesis in response to feeding must elicit efficient re-synthesis of muscle proteins to prevent net protein breakdown and maintain skeletal muscle mass³⁰. Considering the latter, if basal rates of skeletal muscle protein synthesis are similar in obese mice when compared to normal mice, and obese mice synthesize less skeletal muscle protein in response to nutrient ingestion (as shown), there must be an adaptive mechanism(s) for protein sparing in this model otherwise net protein breakdown would occur. The last point is somewhat supported by the fact that we did not observe major differences in lean body mass.

Hepatic insulin resistance has been reported in rodent models following high-fat feeding. For example, Park *et al*³¹ demonstrated that high-fat feeding to C57BL/6J mice resulted in insulin resistance in liver (and skeletal muscle and adipose tissue) after only 3 weeks of dietary intervention. In addition, Samuel *et al*³² found impaired hepatic insulin action after 3 days of high-fat feeding in rats. Since albumin synthesis is sensitive to insulin³³, one might predict a substantial alteration in liver/albumin synthesis in HF fed animals. However, we did not observe any apparent defects in the activation of total liver protein synthesis or plasma albumin in the fed state (Figure 2, Panel B and C, respectively). We suspect that the apparent “normal response” of hepatic protein synthesis may be related to the nature of our experimental design *vs.* that used in insulin-clamp studies. For example, in our study a fairly large bolus of food was given (equal to ~ 35% of the daily intake), which could be sufficient to overcome more subtle defects that can be detected using the insulin-clamp; in addition, nutrients (e.g. amino acids) can stimulate translation independent of insulin. We believe that this should not be construed as a weakness in our data, since one could argue that our design is somewhat more physiological than that which is achieved using the insulin-clamp. More importantly, the apparent discrepancies between our data and those in the literature suggest the need for additional studies in this area and the need to determine the contribution of direct and/or indirect factors, e.g. a primary impairment in insulin action *vs.* a primary alteration in the response of the beta-cell to secrete insulin and/or a primary defect in digestion/absorption. Last, our data suggest a tendency for reduced synthesis of total hepatic proteins and plasma albumin in the basal (fasting) state. These observations may be related to a modest increase in fat deposition in the liver (not shown) and suggest the importance of studies that more closely examine whether the magnitude and/or the duration of fatty liver impacts the synthesis/secretion of hepatic protein(s).

To this point we have considered the nutritional-physiological model and the meaning of the biological data, however, another important area concerns the methodology that we used to quantify protein synthesis. For example, labeled water was used in a few early studies of protein dynamics³⁴⁻³⁶, we have recently revisited the use of $^2\text{H}_2\text{O}$ ^{17;20;37} and H_2^{18}O (unpublished observations). The $^2\text{H}_2\text{O}$ method is based on establishing a precursor:product relationship, i.e. following the administration of $^2\text{H}_2\text{O}$ one determines the rate of protein synthesis by measuring the incorporation of ^2H -labeled alanine into a protein(s) of interest. In our studies we have assumed that the precursor labeling is that of body water and that the product labeling is that of protein-bound alanine divided by 3.7, and that there is rapid equilibration between hydrogen in body water and free alanine. Our previous experiments, and data in the literature, validate these assumptions. First, studies of enzyme reaction mechanisms demonstrate that it is not possible to exchange all four of the carbon-bound hydrogens of alanine^{38;39}, our findings and the recent study by Belloto *et al*⁴⁰ agree with those reports. Second, tracer studies using various isotopes have demonstrated that alanine flux is rapid⁴¹⁻⁴³, for example, the pool turnovers over several times per hour in humans. Again, our experiments in humans²⁰ and rodents¹⁷ are consistent with those data and with the fact that alanine is a central metabolic intermediate. Last, other recent reports have examined the use of $^2\text{H}_2\text{O}$ to measure protein synthesis *in vivo*^{40;44}. Those investigators have also concluded that measuring the incorporation of ^2H -labeled alanine into proteins provides a reliable measure of protein synthesis, even when comparing the $^2\text{H}_2\text{O}$ method against the steady-state leucine infusion⁴⁰.

Although there is a slight discrepancy in the literature regarding the total number of exchangeable hydrogens in free alanine^{17;20;40;44}, for example, Hellerstein and colleagues found that the labeling of alanine is four times that of water⁴⁴, one could argue that the critical factors are (i) the stability of this number over the time course of a study and (ii) whether perturbations affect the value. As demonstrated by our group^{17;20} and Beylot and colleagues⁴⁰, it appears that the ^2H -labeling of alanine is remarkably stable (albeit at ~ 3.7 times that of body water) and that the labeling of plasma alanine reflects that in different tissues.

We believe that the use of $^2\text{H}_2\text{O}$ offers an advantage over the use of labeled leucine, especially in studies where the experimental design involves non-steady state metabolism. For example, a major concern in tracer studies is the stability of the precursor labeling, especially over a prolonged period of time and/or during conditions where substrate flux may suddenly change, e.g. after a meal. Thus, the use of labeled leucine infusion would have been somewhat problematic in our studies since animals would have required catheters to maintain a constant tracer infusion and the bolus of food would have affected the precursor labeling⁴⁵. Perhaps the “flooding dose” would have been reasonable since the expansion of the amino acid pool *via* the flood of tracer might have minimized any perturbation that occurred *via* digestion/absorption of dietary protein. However, a potential concern regarding the flooding dose centers on the duration of the study, i.e. $^2\text{H}_2\text{O}$ permits experiments over a broad window therein allowing one to capture and integrate more metabolic activity *vs.* the flooding dose which is typically used over ~ 60 min (and in some cases only ~ 10 min). We previously demonstrated that in short term studies (e.g. those lasting several hours) it is possible to simply administer an intraperitoneal priming bolus of $^2\text{H}_2\text{O}$ and therein maintain a steady state of labeling¹⁷. For example, since the $t_{1/2}$ of ^2H in body water in mice (fed the respective diets described herein) is ~ 2.5 days²⁶, if the initial target enrichment is 2.5% ^2H -labeling then after 5 hours one expects to find a $\sim 6\%$ decrease in the ^2H -labeling of body water, i.e. from $\sim 2.50\%$ to $\sim 2.35\%$. A recent study by our group used $^2\text{H}_2\text{O}$ to measure protein synthesis in the heart in rats fed various diets, including some with a high salt regimen⁴⁶ which is known to increase water turnover. In that study we were able to obtain serial measurements of water labeling over ~ 6 hours. Although we could detect a slight difference in the ^2H -labeling of samples obtained at 60 min *vs.* 300 min post-injection ($\sim 5\%$ decrease), we could not detect differences between the groups, implying a highly stable precursor labeling within and between groups.

In summary, diet-induced obesity in C57BL/6J mice appears to alter normal protein dynamics. This model may prove useful in future studies since one can readily induce-reverse disease *via* dietary manipulation. Also, since tissue biopsies can be obtained one should be able to identify molecular mechanisms that explain various findings. Although our data differ in regards to some results that have been obtained using insulin-clamps, it is important to note that our studies employed a design that yields an integrative response whereas “clamp studies” allow one to focus on a specific component of the physiological response; these two approaches complement each other. Finally, since $^2\text{H}_2\text{O}$ is suitable for use in humans, it is possible to translate observations between clinical and basic science, of course, studies of albumin synthesis are more practical since an examination of muscle protein synthesis requires tissue biopsies.

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Figure 1. Energy intake and growth

Male C57BL/6J mice were allowed *ad libitum* access to either a low-fat, high-carbohydrate diet (LF) or a high-fat, low-carbohydrate diet (HF). Energy intake (Panel A) was determined by weighing the food that remained on each cage at the end of every week and dividing by 4, i.e. the number of mice per cage. The body weight (Panel B) of individual mice was determined at the end of each week. The data are expressed as mean $\pm$ sem, $n = 16$ per diet group per time point (* $p < 0.05$).

Figure 2. Effect of diet-induced obesity on protein synthesis

Mice were maintained on either a low-fat (LF) or a high-fat (HF) diet for 9 weeks. Protein synthesis was quantified following the injection of $^2\text{H}_2\text{O}$ via the incorporation of ^2H -labeled alanine, mice were studied during either the fasted (saline gavage) or the fed (liquid meal) state. Muscle, liver and plasma albumin synthesis were quantified (Panel A, B and C, respectively). The data are expressed as the % newly made protein per hour, mean $\pm$ sem (n = 6 to 8, muscle and liver data and n = 3 to 4, albumin data), * p < 0.05 fed vs. fasted, # p < 0.05 fed vs. fed.